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CNS of *Drosophila*

I. Identification of the Required Gene Functions

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In the ventral nerve cord of *Drosophila* most axons are organized in a simple, ladder-like pattern. Two segmental commissures connect the hemisegments along the mediolateral and two longitudinal connectives connect individual neuromeres along the anterior–posterior axis. Cells located at the midline of the developing CNS first guide commissural growth cones toward and across the midline. In later stages, midline glial cells are required to separate anterior and posterior commissures into distinct axon bundles. To unravel the genes underlying the formation of axon pattern in the embryonic ventral nerve cord, we conducted a saturating ethylmethane sulfonate mutagenesis, screening for mutations which disrupt this process. Subsequent genetic and phenotypic analyses support a sequential model of axon pattern formation in the embryonic ventral nerve cord. Specification of midline cell lineages is brought about by the action of segment polarity genes. Five genes are necessary for the establishment of the commissures. In addition to *commissureless*, the *netrin* genes, and the netrin receptor encoded by the *frazzled* gene, two gene functions are required for the initial formation of commissural tracts. Over 20 genes appear to be required for correct development of the midline glial cells which are necessary for the formation of distinct segmental commissures. © 1999 Academic Press

INTRODUCTION

Neurons send out axonal processes to form a highly ordered lattice of intercellular connections. To date a number of different mechanisms have been described which are used by the growing tip of the axon, the growth cone, to navigate through different tissues to finally reach its destination (Goodman and Shatz, 1993; Tessier-Lavigne and Goodman, 1996). But what are the genes encoding the signals and the receptors utilized by the growth cone to precisely reach and contact its target? This question has been addressed in several animal systems by a number of techniques. A biochemical approach in chicken resulted in the discovery of the netrin proteins required for guidance of commissural growth cones toward the floor plate in the vertebrate neural tube (Kennedy *et al.*, 1994; Serafini *et al.*, 1994). Interestingly, the vertebrate genes encoding netrin and different netrin receptors (*dcc* and *rcm*) turned out to be homologous to genes involved in circumferential migration in *Caenorhabditis elegans* (*unc*-6, *unc*-5, and *unc*-40) (Ackerman *et al.*, 1997; Chan *et al.*, 1996; Fazeli *et al.*, 1997; Hamelin *et al.*, 1993; Hedgecock *et al.*, 1990; Keino-Masau *et al.*, 1996; Leonardo *et al.*, 1997). These genes, as well as the *Drosophila unc-40* homologue *frazzled*, were found in diverse genetic screens (Hedgecock *et al.*, 1985; Kolodziej *et al.*, 1995).

The strength of the genetic dissection of any complex biological process lies in the fact that it is an unprejudiced view. In their classical studies Nüsslein-Volhard and Wieschaus (1980) deduced the principles of early pattern formation in the *Drosophila* embryo based solely on the different mutant phenotypes they identified. Since then, the genetic approach has been used in several other organisms to dissect other developmental processes (Driever *et al.*, 1994; Hedgecock *et al.*, 1985; Mayer *et al.*, 1991; Mullins and Nüsslein-Volhard, 1993). Genetics intends to eventually provide answers to questions like: what are the fundamental developmental mechanisms? The mutant phenotypes reveal the parameters underlying any specific developmental process. How complex is the regulation of a given process? The pattern of complementation, along with the phenotypic analysis, reveals the numbers of developmental steps and of genes required at each step. Finally, the analysis of double and triple mutant embryos allows one to define functional interactions between the different genes and thus the network of genetic functions underlying the process under study.

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The central nervous system (CNS) of *Drosophila* provides an attractive model system for an unbiased genetic approach aimed at unraveling the genes required for axon pattern formation since its morphology has been extensively studied (Goodman and Doe, 1993). About 350 neurons and glial cells found in each abdominal hemineuromere of the *Drosophila* ventral nerve cord stem from approximately 30 neuroblasts (Bossing *et al.*, 1996; Schmidt *et al.*, 1997). The majority of the CNS neurons are interneurons projecting their axons to the contralateral side in one of the two segmental commissures. All these neurons have to fulfil a very similar task. Initially, contralaterally projecting growth cones have to be attracted by CNS midline cells. However, once these growth cones have reached and crossed the midline they change their growth behavior and extend away from the midline to continue to grow along longitudinal pathways which they ignored on the ipsilateral side.

The importance of the CNS midline, which was first noticed by Escherich, is a general feature of bilateral symmetric nervous systems (Escherich, 1902). In *Drosophila* the CNS midline comprises a small set of glial and neuronal cells: three or four midline glial cells, two MP1 neurons, six VUM neurons, two UMI neurons, and the median neuroblast and its support cells (Bossing and Technau, 1994; Jacobs and Goodman, 1989; Klämbt *et al.*, 1991). Similar to the floor plate cells in vertebrates, the *Drosophila* midline cells are the first CNS cells specified (Cres *et al.*, 1988; Schoenwolf and Smith, 1990). In *Drosophila* their development is under the control of the PAS domain transcription factor single-minded, forming heterodimers with the tango cofactor (Ohshiro and Saigo, 1997; Sonnenfeld *et al.*, 1997). Ectopic expression of *single-minded* within the ectoderm directs the formation of additional midline cells (Nambu *et al.*, 1991). Depending on where *single-minded* is expressed within a segment, midline neurons or midline glial cells develop (Menne *et al.*, 1997).

The subsequent development of some of the midline neurons depends on the gene *orthodenticle (otd). otd* is expressed in the VUM neurons, some of which are missing in *otd* mutant embryos (Finkelstein *et al.*, 1990). The function of the midline neurons during development of the axonal pattern has not been addressed in much detail so far. The midline glial cells function during commissure maturation, when two midline glial cells migrate along cell processes of the VUM neurons to separate anterior and posterior axon commissures (Klämbt *et al.*, 1991). Later, the midline glial cells are required for the formation of individual fascicles within the commissures (Stollewerk and Klämbt, 1997).

The efficacy of the genetic analysis depends on the phenotypic traits that can be detected. In the case of the development of the nervous system, antibodies have proven to be extremely powerful in revealing a number of those phenotypic traits. As different antibody probes and enhancer trap markers became available to visualize axonal patterning, genetic screens were employed to analyze the development of various parts of the nervous system, i.e., the visual system, the motoneurons, the peripheral nervous system, and the central nervous system axon pattern (Kolodziej *et al.*, 1995; Martin *et al.*, 1995; Salzberg *et al.*, 1994; Schmucker *et al.*, 1997; Seeger *et al.*, 1993; van Vactor *et al.*, 1993). However, despite all these efforts many questions remained open in the genetic analysis of neural development.

Over the past several years we conducted a saturating EMS mutagenesis aimed at identifying all zygotically active genes required for the correct development of the axon pattern in the ventral nerve cord in the *Drosophila* embryo. Here we present the results of a comprehensive study of gene functions required during different phases of commissure formation. Following the formation of the midline cells and their subsequent specification, the initiation of commissure development takes place, which is orchestrated by only a few genes. Final shaping of anterior and posterior commissures, however, appears to depend on a surprisingly high number of gene functions. We show an important contribution of the midline neurons in attracting commissural growth cones. The results presented are finally integrated in a model on commissure development in *Drosophila*.

EXPERIMENTAL PROCEDURES

Genetics

Identification of mutations affecting axon pattern formation. In order to identify all zygotically active genes which are required for normal CNS axon pattern development we mutagenized isogenic males with 25 mM ethylmethane sulfonate (EMS) (Lewis and Bacher, 1968). The following genotypes were mutagenized: X chromosome, *y f v mal;* second chromosome, *cn bw;* and third chromosome, *st e*. About 50% of the third chromosomal mutations were generated on a *st AA142* P[*white, lac*Z] *e* chromosome. This chromosome carries an enhancer trap insertion associated with β-galactosidase expression in the midline glial cells (Klämbt *et al.*, 1991). We established 37,300 lines, 18,290 of which carried at least one lethal hit. Mutant chromosomes were kept over "blue balancers." Assuming a Poisson distribution, about 30,000 lethal hits were generated and screened (see Table 1 for details).

Screening for mutations affecting CNS development. All lethal lines were analyzed for phenotypic abnormalities following whole-mount antibody staining using monoclonal antibodies (MAb) BP102 and 22C10 (Fujita *et al.*, 1982). In addition, we simultaneously used polyclonal antisera against β-galactosidase (Cappel) to unambiguously identify embryos carrying "blue" balancer chromosomes. To process whole-mount antibody staining from many genotypes we used a rapid egg collection and staining device (Hummel *et al.*, 1997). Embryos from each line were initially analyzed under the dissecting microscope and axonal defects were subsequently characterized using a Zeiss Axiophot microscope.

Of the lethal lines, 6.66% (1218 of 18,290) showed nervous system defects. Neural phenotypes accompanied by gross morphological abnormalities were considered "nospecific," leaving 757 lines with a "specific" nervous system defect (see Table 1 for details). The mutations used are listed in Table 2. In addition to the

TABLE 1
Summary of the Genetic Screen

	X chromosome	2nd chromosome	3rd chromosome	Total
Established lines	19,300	10,200	7,800	37,300
Lethal lines	5,810	5,980	6,500	18,290
Lethal hits[a]	6,912	9,002	13,976	29,890
With neuronal phenotype	346	468	404	1,218
Kept lines	215	280	262	757

Note. The number of alleles isolated is indicated.
[a] The number of lethal hits was calculated assuming a Poisson distribution.

EMS-induced mutations we determined the axonal phenotypes associated with the available chromosomal deficiencies. With regard to the formation of the CNS axon pattern the deficiency *Df*(1)*RK*2 leads to a phenotype for which no EMS-induced mutations were found. This deficiency removes the two redundant *netrin* genes (Harris *et al.,* 1996; Mitchell *et al.,* 1996).

The enhancer trap lines *AA142, AE60,* and *X55* were used to characterize different midline cells (Klämbt *et al.,* 1991). *H90* is a lethal insertion into the *hedgehog* gene; *H40* is a lethal insertion in the *patched* gene (unpublished).

Immunohistochemistry

Embryos were collected and stained as described previously (Hummel *et al.,* 1997).

RESULTS

Embryonic CNS Axon Pattern

The embryonic axon pattern in the ventral nerve cord develops in sequential steps in close relationship with midline glial and neuronal cells (Fig. 1) (Bossing and Technau, 1994; Jacobs and Goodman, 1989; Klämbt *et al.,* 1991). The first growth cones navigate toward the anterior most VUM cell and thus pioneer the prospective posterior commissure. Only when the posterior commissure is established, the anterior commissure forms. Now the midline glial cells (blue in Fig. 1) start to migrate posteriorly to separate anterior and posterior commissures (Klämbt *et al.,* 1991). The VUM neurons reside ventral to the posterior commissure and project in a characteristic axon-bundle to the anterior commissure (Fig. 1D, open arrowheads). Migration of two midline glial cells occurs along these cell processes.

To visualize the axonal structures as described above we used two different monoclonal antibodies to analyze the CNS axon pattern in the screening process. In order to proceed through large numbers of mutagenized chromosomes we developed an egg collection/embryo staining device, which allows the simultaneous processing of more than 600 genotypes per day (Hummel *et al.,* 1997). About 50% of all the third-chromosomal mutations were directly screened for alterations in the number and position of the midline glial cells highlighted by the *AA142* enhancer trap activity (Figs. 1B–1D).

Phenotypic Classes

After screening more than 18,000 lethal lines we found 757 mutations leading to a specific nervous system defect. Of these mutations 521 (about 70%) predominantly affect the development of the CNS. Four hundred thirty were subsequently grouped according to their axon pattern phenotype into seven major classes. The remaining mutations led to subtle CNS phenotypes and thus were so far not placed into any group. A major class comprises mutations affecting the formation of both commissures and connectives (24% of the 430 mutations). Therefore, the corresponding genes exert a more general effect on either neuronal specification or axon outgrowth (e.g., *redax* mutation in Fig. 2B). So far known genes in this class are *propero* (eight alleles), *pavarotti* (five alleles), and *cyclin A* (two alleles). About 25% of the identified mutations lead to a reduction in the number of longitudinal axon tracts. For example, we found five new *lola* alleles (Seeger *et al.,* 1993; Giniger *et al.,* 1994) and two *sanpodo* alleles (Salzberg *et al.,* 1992; Dye *et al.,* 1998). An extreme case is a complete loss of all longitudinal connectives as seen in mutant *noco* embryos (Fig. 2C). These classes will not be discussed further.

Mutations affecting the formation of commissures. Mutations in four genes primarily affect the formation of commissures. For example, in *commissureless* mutant embryos no commissures can be detected at all, whereas longitudinal connectives remain intact (Figs. 2D and 5; see below).

Mutations leading to a collapse of all CNS axon tracts. A collapse of all longitudinal axon tracts at the midline is observed in *single-mineded (sim)* mutant embryos and, subsequently, no commissures are detected (Figs. 2E and 2I). In mutant *single-minded* embryos all midline cells degenerate. We also observed frequent breaks in the longitudinal connectives as well as a defect in nerve cord retraction. In addition to *single-minded,* mutations in three other genes result in a similar CNS midline phenotype (Fig. 3, discussed below).

TABLE 2
Genes Identified in the Screen

Gene		Number of alleles	Location	Reference
cabrio	*cao*	2	2-20 (26D)	This work
commissureless	*comm*	2	71E3-5	1, 2
engrailed	*en*	3	48A3-4	3
faint little ball	*top*	11	57F1	4
frazzled	*fra*	1	49B1-12	5
hedgehog	*h h*	3	94E2	3
jaywalker	*jay*	2	3-44 (+/−2)	6, this work
karusell	*kus*	4	1-55 (+/−2)	This work
kästchen	*kas*	4	3-26 (+/−1)	This work
kette	*kte*	5	3-47 (79E)	This work
klötzchen	*klo*	4	3-2 (+/−2)	This work
kübel	*kub*	3	1-42	This work
möchtegern	*mog*	2	3-62 (+/−2)	This work
orthodenticle	*otd*	5	8A1-12	7, 8
patched	*ptc*	2	44D3-9	3
pointed	*pnt*	7	94E/F	9, 10
rhomboid	*rho*	5	62A1-3	9
roundabout	*robo*	4	58F3	1, 11
schizo	*siz*	2	3-51 (+/−3)	This work
schmalspur	*sur*	3	3-40 (+/−2)	This work
single-minded	*sim*	5	87E1	12
slit	*sli*	10	52D1-9	4
spitz	*spi*	2	37F4-5	9
Star	*S*	4	21E2	9
weniger	*weg*	4	1-0 to 1-1.5	This work
perle	*pel*	1	3-26	This work
shroud	*sro*	2	3-100	4
spook	*spo*	4	3-19	4
disembodied	*dib*	4	3-12	4
shadow	*sad*	4	3-51	4
shade	*shd*	3	3-41	3
phantom	*p h m*	6	1-60	8
rippchen	*rip*	3	3-44	This work
wingless	*wg*	5	28A1	3

Note. The number of alleles isolated is indicated. Newly identified mutations have been mapped meiotically. *cabrio* and *kette* were mapped cytologically by deficiency mapping and additional P-element-induced alleles. References: (1) Seeger *et al.* (1993). (2) Tear *et al.* (1996). (3) Nüsslein-Volhard and Wieschaus (1980). (4) Nüsslein-Volhard *et al.* (1984). (5) Kolodziej *et al.* (1996). (6) Salzberg *et al.* (1994). The mapping of *jaywalker* was performed in this work. (7) Finkelstein *et al.* (1990). (8) Wieschaus *et al.* (1984). (9) Mayer and Nüsslein-Volhard (1988). (10) Klämbt (1993). (11) Kidd *et al.* (1998). (12) Thomas *et al.* (1988). Explanation of the German names: *karussell,* merry-go-round; *kästchen,* small box; *kette,* chain; *klötzchen,* small building block; *kübel,* bucket; *möchtegern,* would like to be; *perle,* pearl; *rippchen,* small rib; *schizo,* split; *schmalspur,* narrow gauge; *weniger,* less.

Mutations affecting the separation of commissures. In 114 mutations the segmental commissures do not become separated into anterior and posterior axon bundles and appear fused (i.e., mutant *Star* embryo, Figs. 2F and 2K). So far 76 mutations which give rise to such a phenotype were placed in 20 complementation groups and are discussed below. Mutations in *roundabout* and *karussell* were initially placed into this group but were later found not to affect the separation of commissures but to be components of the repulsive system (Hummel *et al.,* 1999).

Mutations affecting fasciculation within the commissures. Fifty-six mutations lead to a CNS axon tract phenotype which superficially looks similar to the fused commissure phenotype. This is the "fuzzy" commissure phenotype. In the most severe expression, no space remains between anterior and posterior commissures. However, during development the commissures initially become separated and only during later stages (stage 15) are axons found in a criss-cross pattern at the midline (Figs. 2G and 2L). This is never observed even in hypomorphic mutations of the fused commissure group genes. We interpret the fuzzy commissure phenotype as a consequence of a late midline glial cell defect or of defects in fasciculation of commissural axons. A detailed analysis has not yet been performed.

Mutations leading to novel phenotypes. We identified only one X-chromosomal complementation group with a CNS phenotype which could not be placed in any of the above classes. In *chaos (chs)* embryos, commissures and connectives form; but in a sometimes aberrant manner (Figs. 2H and 2M). In some instances we observed neuromeres with three commissures (arrow in Fig. 2M). the "normal" posterior commissure is marked by the projection of the VUM neurons (Fig. 2M, open arrowhead). In other segments, commissures do not cross the midline orthogonally as in wild type, but instead appear to follow the diagonally projecting axons of the VUM midline neurons (Fig. 2M, arrowheads).

The observed phenotypes indicate that the stepwise development of commissures and connectives is controlled by different, only partially overlapping gene functions. In the following we concentrate on genes required early during the formation of the commissural axon pattern. Later aspects of commissure development as well as the dissection of midline glial cell functions are presented in a second paper (Hummel *et al.,* 1999).

Formation of Midline Cells

We were interested in correlating the different mutant axonal phenotypes to specific defects in the CNS midline and subsequently to the sequential development of the CNS axon pattern (Klämbt *et al.,* 1991). In a first step the CNS midline cells need to be specified. Previous work has determined the important function of *single-minded* as a master regulatory gene of midline development. Mutations in *single-minded* (*sim;* 5 alleles found), *slit* (*sli;* 10 alleles found), and *faint little ball* (*flb;* 11 alleles found) all display a collapsed CNS axon phenotype with frequent breaks in the longitudinal connectives (Figs. 1E, 3A and 3B). Mutant *jaywalker (jay)* embryos (Salzberg *et al.,* 1994) display a

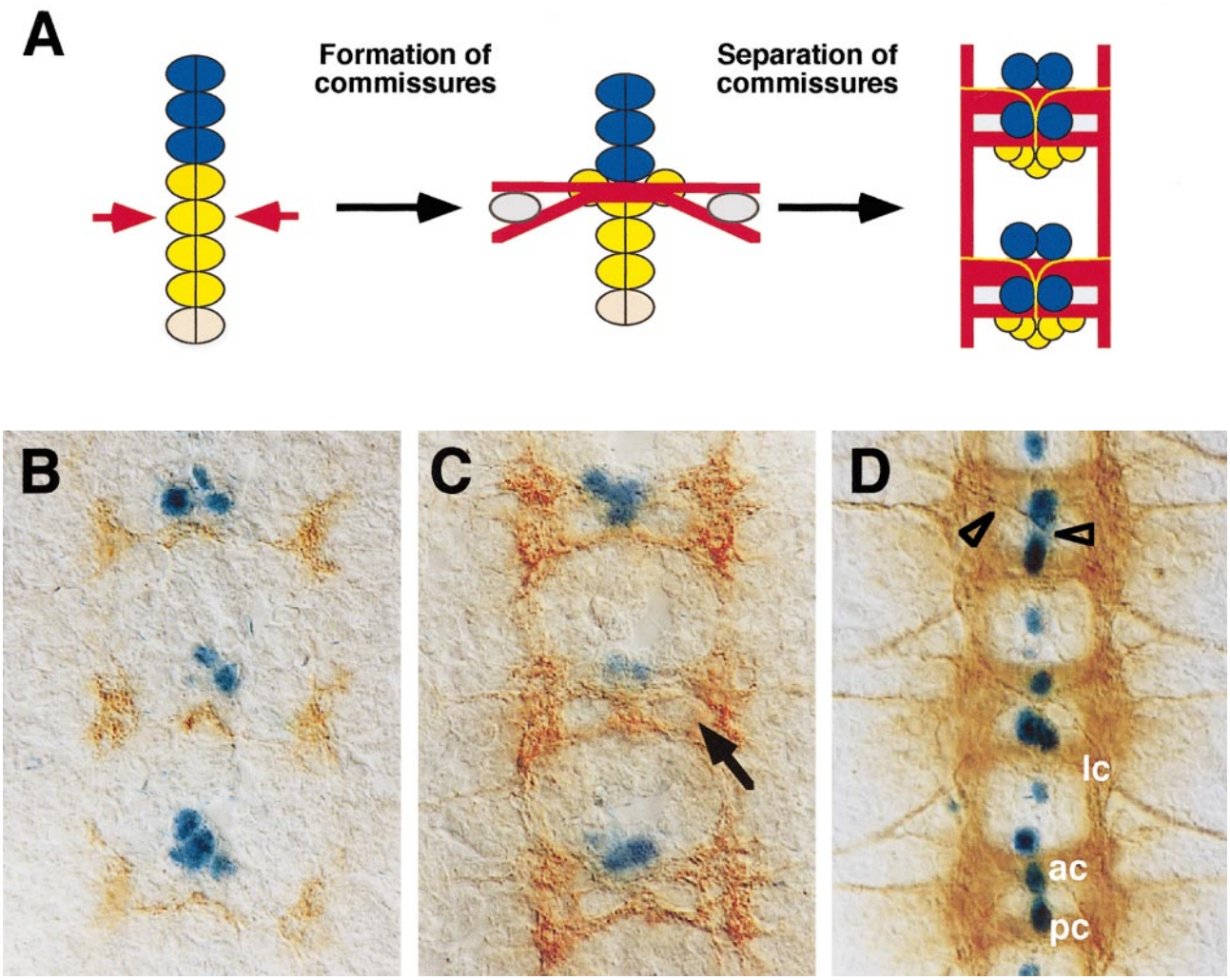


FIG. 1. The development of the embryonic CNS axon pattern. (A) Schematic view of the sequential steps of commissure formation. The midline glial cells are shown in blue. The VUM neurons are in bright yellow, the median neuroblast is in pale orange, the RP1 neurons are indicated in gray. Axons are shown in red, anterior is up (B–D) Frontal view of dissected embryonic CNS preparations. The CNS axons are highlighted using the MAb BP102 and subsequent HRP immunohistochemistry (brown). The midline glial cells express β-galactosidase (blue) driven by the *AA142* enhancer trap insertion (Klämbt *et al.*, 1991). (B) The development of the commissures starts in early stage 12 when the later posterior commissure is formed. The first commissural growth cones grow toward the anteriormost VUM neurons, the midline glial cells (blue) are located anterior to where the commissural axons cross the midline. (C) Once the posterior commissure is established, the anterior commissure forms in its immediate vicinity. Two cell movements can be detected. The RP1 motoneurons (arrow) move medially and the midline glial cells move posteriorly. (D) The two segmental commissures are brought into the final ladder-like arrangement by an interacalating migration of the midline glial cells along axons of some VUM neurons (open arrowheads). The RP1 neurons are now in their final position close to the midline. Once the segmental commissures are established, the longitudinal connectives are formed. ac, anterior commissure; pc, posterior commissure; lc, longitudinal connective.

weak *single-minded*-like phenotype. Here, the connectives are collapsed and reduced as in *single-minded* but appear less compacted at the level of the commissures (Fig. 3C).

Using a *sim-lacZ* construct, the number and position of all CNS midline cells can be determined (Nambu *et al.*, 1990) (Figs. 3D and 3E). With this construct, we observed a slight reduction in the number of midline glial cells in mutant stage 10 *flb* embryos; however, during stage 12 the *sim-lac*Z-positive cells were displaced ventrally and could not be detected any more in stage 16 embryos (Fig. 3F). In this stage, only one to three midline cells per neuromere expressed the *sim-lac*Z marker (data not shown). In *flb* or *single-minded* mutant embryos, midline cells degenerate; in *slit* mutant embryos, however, midline cells are specified correctly but become displaced ventrally during stage 12 onward (Sonnenfeld and Jacobs, 1994) (Fig. 3G). In mutant

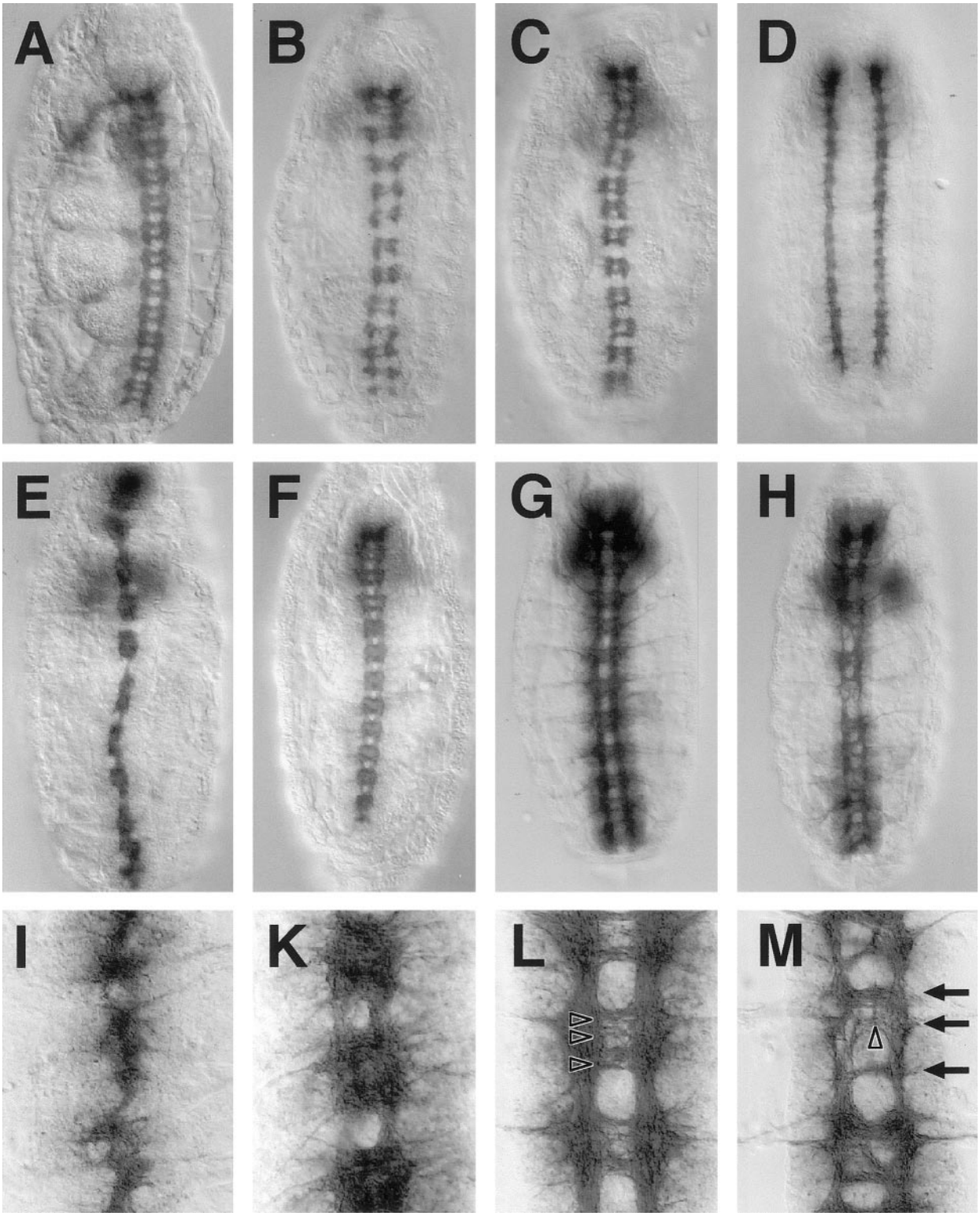


FIG. 2. Summary of different phenotypic classes identified in the screen. (A–H) Whole-mount preparations of stage 15/16 embryos. (I–M) Frontal views of stage 15/16 CNS preparations. The CNS axon tracts have been labeled by MAb BP102 and subsequent HRP reaction. Anterior is up. (A) In a wild-type embryo CNS axons are arranged in a regular ladder-like pattern. (B) In *redax* mutations a severe reduction

jaywalker embryos midline cells are specified normally as seen with the *sim-lac*Z reporter construct. In stage 9 embryos we observed a conspicuous folding of the ventral ectoderm (Fig. 3H). Some midline cells appeared to be missing or were displaced ventrally from stage 12 onward (Fig. 3I). Using the midline neuron marker *X55* and the midline glia marker *AA142*, both midline lineages appeared to be affected (data not shown).

Specification of CNS Midline Cells

In the next step the individual CNS midline cell types must be specified. What determines the number of midline neurons versus midline glial cells? Since many of the third chromosomal mutations were generated on a chromosome marked with the *AA142* enhancer trap insertion, the number of midline glial cells in the different mutations was determined during the screening process.

Mutations leading to early alterations in the number of midline glial cells were found to be allelic to previously known segment polarity mutations. Mutant *hedgehog* embryos were initially identified based on a dramatic increase in the number of midline glial cells (stage 13; Fig. 4A). Here we found >16 midline cells per neuromere expressing the *AA142* enhancer trap marker compared to 4–6 cells in wild-type neuromeres. All *AA142*-positive cells are found in dorsal positions within the ventral nerve cord, a position characteristic for midline glial cells. At this stage some of the ectopic midline glial cells are already pycnotic. In stage 14 embryos they are still present; however, they adhere to the gut (Fig. 4B; not shown). By the end of embryogenesis, most of the midline glial cells are missing. Concomitant to the increase in the number of midline glial cells, a reduction in the number of midline neurons was found. In wild-type embryos, the enhancer trap marker *X55* leads to β-galactosidase expression in about 14 midline neurons per neuromere (Klämbt *et al.*, 1991). In *hedgehog* mutant embryos only 2–4 cells per neuromere which express the *X55* β-galactosidase marker remain. Interestingly, *hedgehog* is expressed as *engrailed* in midline neurons (Lee *et al.*, 1992; our own observations using the *H90* enhancer trap insertion into *hedgehog* which mimics the entire *hedgehog* expression pattern).

Commissure formation appears to be delayed in mutant *hedgehog* embryos. In stage 13 only a few commissural axons have crossed the midline (Fig. 4B). In stage 16 mutant *hedgehog* embryos commissures are formed; however, they are reduced in size (Fig. 4D).

Mutations in *patched* lead to complementary alterations in the number of different midline cells. The number of midline cells expressing the *AA142* midline glial cell marker is drastically reduced throughout embryonic development (zero or one cell per neuromere compared to the normal three or four cells per neuromere; Figs. 4E and 4F). Concomitantly, we observed an increase in the number of *AE60*- or *X55*-positive midline neurons (Fig. 4G). In mutant *patched* embryos, commissural growth cones are initially heading toward the CNS midline cells as in wild type; however, once they have reached the midline they stall and cross it only during later stages (stage 13/14; Figs. 4G, 4H, and 4I). No distinct anterior or posterior commissures develop and commissures appear to be fused (Fig. 4F). *patched* is expressed in the nervous system and in many midline cells (Nakano *et al.*, 1989; Hooper and Scott, 1989; our own observations using the enhancer trap line *H40*). The commissural phenotypes observed in *hedgehog* and *patched* mutant embryos could be due to a function of these genes during lateral CNS development (Patel *et al.*, 1989). However, it is unlikely that all contralateral projecting neurons are affected by these mutations, suggesting that the midline defects are at least in part responsible for the observed commissural phenotypes.

Mutations in *patched* and *hedgehog* lead to interpretable phenotypes concerning the alterations in the number of the different midline cell types. In contrast, *wingless* embryos show an increased number of midline glial cells (Fig. 8) but neuronal differentiation markers are still expressed (data not shown). We have not determined whether midline cells coexpress glial and neuronal markers. *wingless* embryos show reduced formation of commissures (Fig. 8). Similarly, *engrailed* affects the formation of midline neurons but does not lead to an expansion of the midline glial cell lineage (data not shown).

Formation of Commissures

Once the midline cells have been specified, they guide commissural growth cones toward and across the midline. Recently the netrin proteins and the netrin receptor encoded by *frazzled (fra)* have been identified in *Drosophila*. Disruption of the genes encoding these components leads to a reduction in the number of commissural axon tracts

in the axonal growth can be detected. Formation of connectives and commissures is affected. (C) In *noco* embryos formation of commissures appears normal, the formation of the longitudinal axon tracts is impaired. (C) In *commissureless* mutant embryos no commissures form. (E and I) In *single-minded* embryos all midline cells degenerate from stage 12 onward and the longitudinal axon tracts collapse at the midline. (F and K) In mutant *Star* embryos the differentiation of the midline glial cells is affected. The segmental commissures do not become separated any more. (G and L) *verfilzt* mutant embryos show a fuzzy commissure phenotype. Here too, axons irregularly cross the space between anterior and posterior commissure (arrowhead). This phenotype, however, develops late after commissures have been separated. (H and M) In mutant *chaos* embryos abnormal commissure formation is observed. In (M) a neuromere with three distinct commissures is shown (arrows). The open arrowhead points toward the medial VUM axons which run between posterior and anterior commissure.

(Harris *et al.*, 1996; Kolodziej *et al.*, 1996; Mitchell *et al.*, 1996). However, neither mutation leads to a complete loss of all commissural axon connections (Fig. 5B; for a *frazzled* loss of function phenotype see Kolodziej *et al.*, 1996; Hummel *et al.*, 1999).

We have identified four complementation groups which lead to a reduced commissure phenotype. Mutations in individual *netrin* genes were not recovered. This is not surprising, since two partially redundant *netrin* genes reside in close proximity on the X chromosome (Harris *et al.*, 196; Mitchell *et al.*, 1996). However, the *netrin* phenotype was identified in the screen of embryos carrying chromosomal deficiencies (Fig. 5B). The phenotypic analysis showed that the posterior commissure is affected most frequently (Fig. 5B). In addition to mutations in the previously known genes *frazzled* (one allele; Fig. 5C) and *commissureless* (two alleles; Fig. 5D), two other genes leading to a reduced commissure phenotype were also identified: *schizo* (two alleles; Fig. 5E) and *weniger* (four alleles; Fig. 5F).

In embryos homozygous for a *frazzled* mutation only a few commissural fibers in the anterior or in the posterior commissures are affected (Fig. 5C, arrows). *schizo* and *weniger* mutant embryos show a more pronounced loss of commissural fibers (Figs. 5E and 5F). Genetic analyses indicate that the *weniger* and *schizo* alleles are at least strong hypomorphs. Embryos mutant for either of the two *schizo* alleles show a variable axon pattern phenotype. Some neuromeres lack all commissural connections, whereas in other neuromeres both anterior and posterior commissures form. In most neuromeres, the anterior commissure is more severely affected (Fig. 5E, arrowhead). In addition, the longitudinal connectives are slightly reduced. Mutations in *weniger* lead to a stronger reduction in the number of commissural fibers (Fig. 5F). In some neuromeres both commissures are absent (Fig. 5F, arrowhead). In contrast to the *schizo* phenotype, the posterior commissure is more often affected. The number of midline glial cells in normal in all reduced commissure mutations during the initial formation of commissures. However, in later stages axon contact is required for the survival of the midline glial cells (Sonnenfeld and Jacobs, 1995). We find that the reduction in the number of midline glial cells correlates with the reduction in the number of axons crossing the midline (Fig. 8).

The putative functions of *roundabout* and *karussell* which are needed for repulsive function of the CNS midline cells are described separately (Hummel *et al.*, 1999).

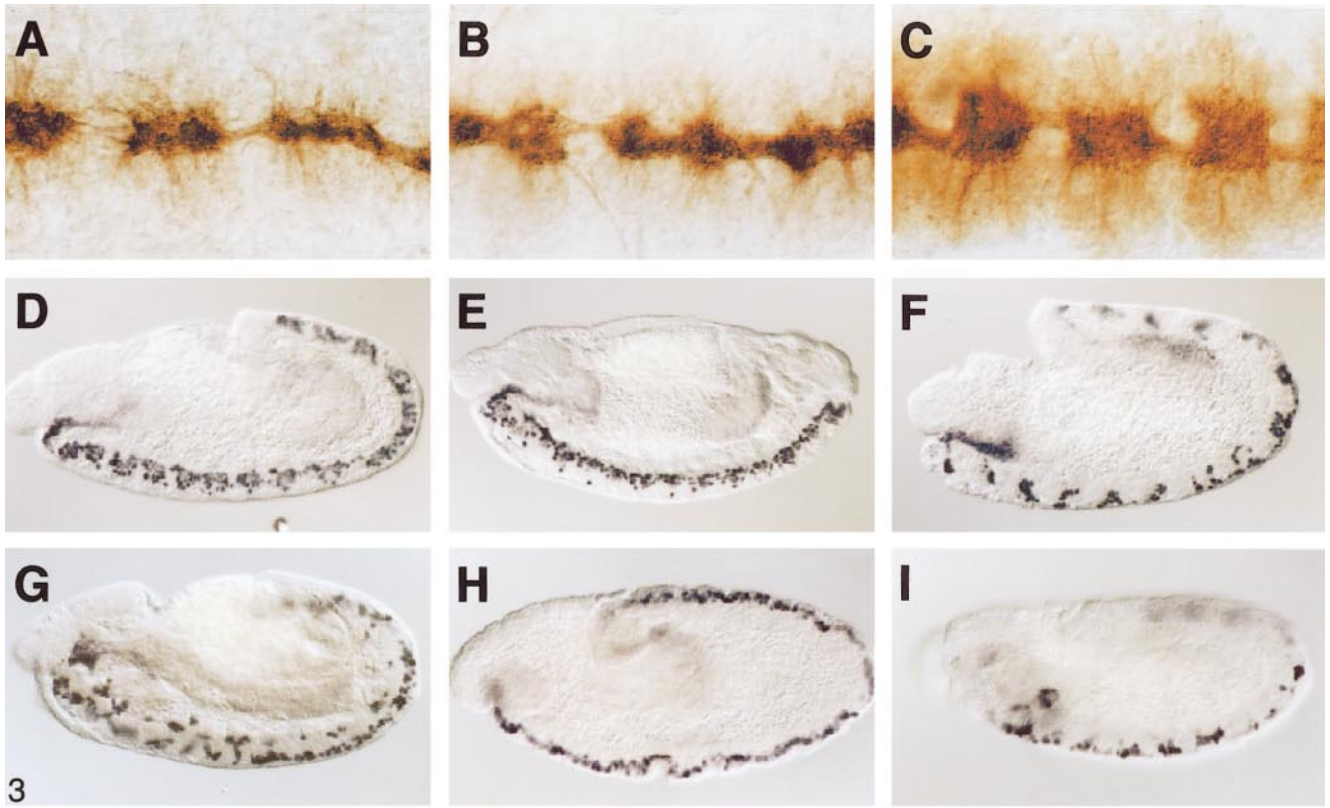
Separation of Commissures

Anterior and posterior commissures initially form in close proximity. Only after an intercalating migration of two midline glial cells are the commissures separated. Disruption of this glial cell migration leads to a "fused commissure" phenotype (Figs. 2F and 2K; Klämbt *et al.*, 1991). We have identified a surprisingly high number of mutations leading to such a phenotype. They define more than 20 genes required for midline glial cell migration. The different fused commissure mutant phenotypes can be placed into two major classes: the *pointed* group encompassing 12 genes and the *tramtrack* group with 8 members. About 20 fused commissure mutations belonging to the *pointed* group have not been characterized yet.

Mutations in 12 genes lead to an axon pattern phenotype similar to that described for some members of the *spitz* group (Klämbt *et al.*, 1991; Mayer and Nüsslein-Volhard, 1988). Since none of the new mutations share the cuticle phenotype which leads to the description of the *spitz* group we placed these genes together with some of the *spitz* group mutants in the so-called *pointed* group (*pointed*, *klötzchen*,

FIG. 3. Midline cells in *single-minded*-like mutations. (A–C) Frontal vies of stage 15/16 CNS preparations. The CNS axon tracts have been labeled by MAb BP102 and subsequent HRP reaction. (D–I) Lateral views of whole-mount preparations stained for the presence of β-galactosidase expression directed by a *sim-lac*Z transgene. Anterior is to the left. Mutations in four genes lead to a collapse of the longitudinal axon tracts at the midline. In addition to *single-minded* (see Fig. 2E), mutations in (A) *faint little ball* and (B) *slit* show a complete collapse of the axon tracts. (C) Mutations in *jaywalker* lead to a weaker phenotype; however, the connectives are always fused. (D) Stage 12 wild-type embryo expressing the *sim-lac*Z trangene, which is expressed in all midline cells. (E) Stage 14/15 wild-type embryo expressing the *sim-lac*Z transgene. (F) In mutant *faint little ball* stage 12 embryos the midline cells are found ventrally. (G) In stage 12 *slit* embryos we observe a similar phenotype. (H and I) Midline cells in *jaywalker* embryos. (H) In stage 9 mutant *jaywalker* embryos a frequent folding of the ventral ectoderm is observed. (I) In later stages, the midline cells are dislocated to the ventral side of the embryo.

FIG. 4. Segment polarity genes affect the midline cell lineages. Frontal views of CNS preparations are shown. The CNS axon tracts have been labeled by MAb BP102 and subsequent HRP reaction (brown). β-Galactosidase expression in different subsets of midline cells is detected by anti-β-galactosidase antibodies and subsequent alkaline phosphatase detection (blue). Anterior is up. (A) In stage 13 mutant *hedgehog* embryos an increased number of midline glial cells expressing the *AA142* marker can be detected (see Fig. 1C for wild-type pattern). (B) In stage 15 *hedgehog* embryos few midline glial cells are present in the CNS. Commissures have not been formed in the normal number. (C) Only two to four midline cells express the *X55* marker in mutant *hedgehog* stage 12 embryos. (D) In stage 16 *hedgehog* embryos still few midline cells express the *X55* marker. In addition some axons have crossed the midline. (E) In stage 13 mutant *patched* embryos almost no *AA142*-positive midline cells can be detected. Arrows point to midline glial cells. (F) In stage 16 *patched* embryos commissures appear fused; only a few displaced *AA142*-positive midline glial cells can be detected (arrows). (G) Almost all midline cells express the *X55* marker in a stage 12 mutant *patched* embryo. (H) In stage 12 mutant *patched* embryos commissural axons grow normally toward the midline but appear to stall at the midline (arrows). (I) Finally, axons cross the midline.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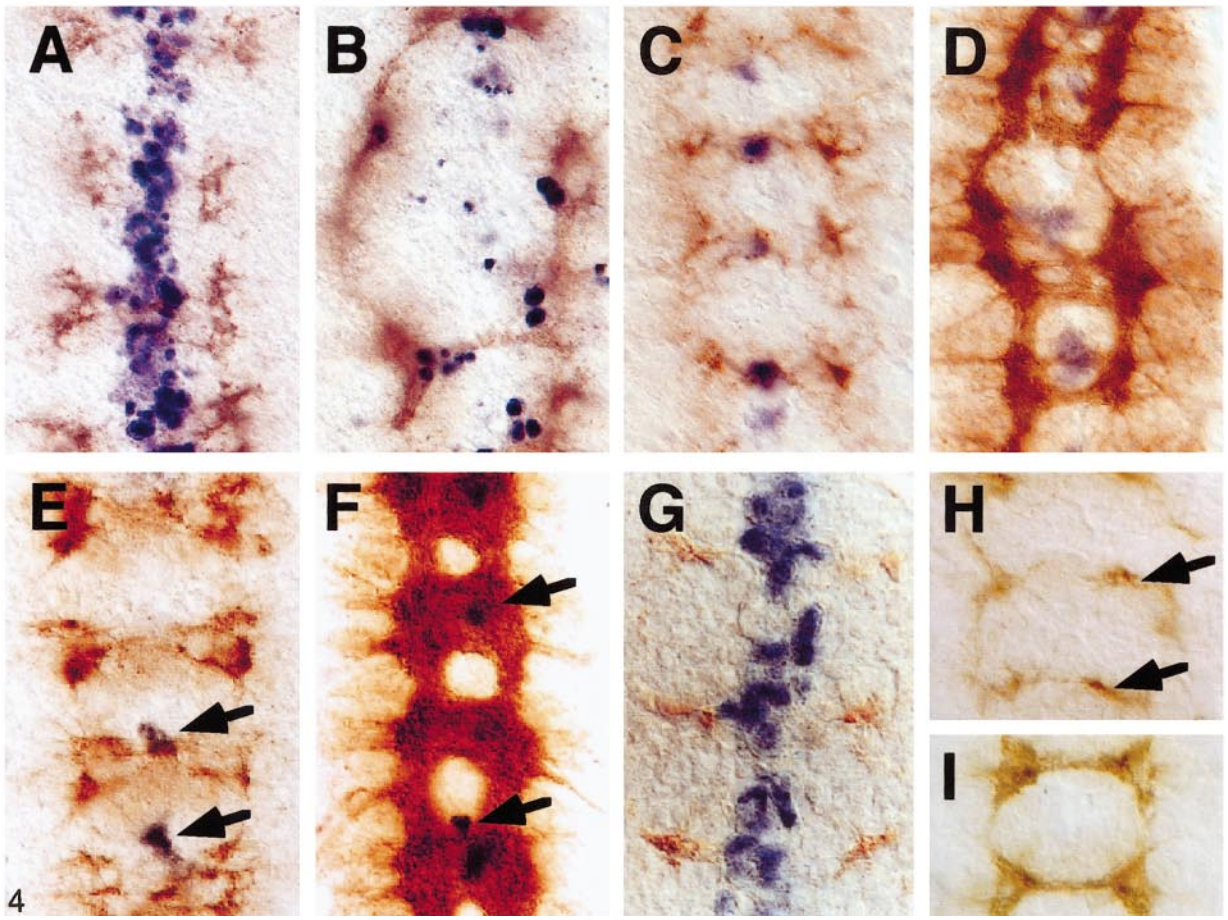
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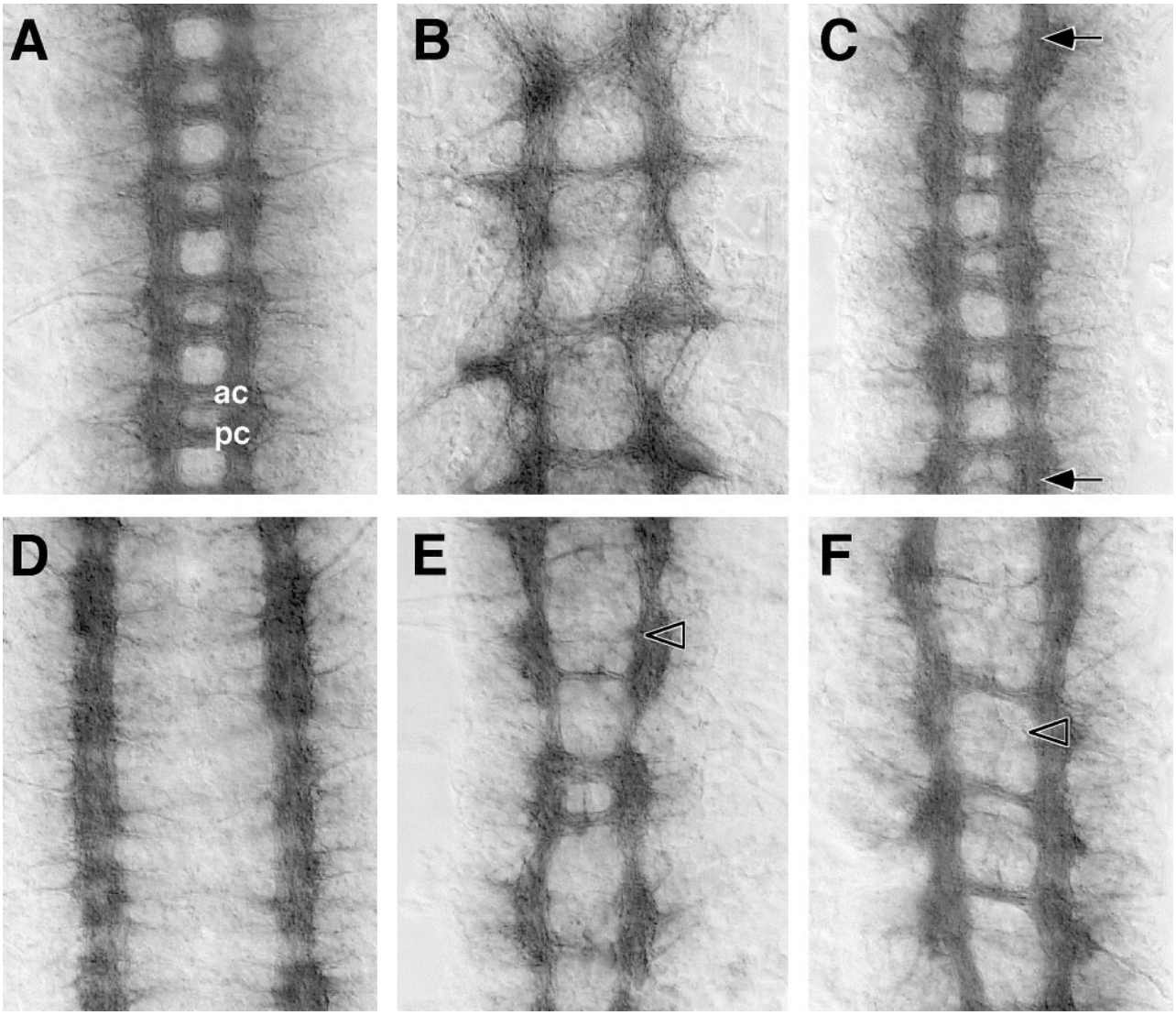


FIG. 5. Few genes lead to a reduction in the number of commisural axon tracts. Frontal views of stage 15/16 CNS preparations are shown. The CNS axon tracts have been labeled by MAb BP102 and subsequent HRP reaction. Anterior is up. (A) Wild-type embryo, anterior and posterior commissures are indicated (ac, pc). (B) The deficiency *Df(1)RK2* leads to a marked reduction in the number of commissural axons crossing the midline. Note that some axons still cross the midline in an anterior-like commissure. (C) In embryos homozygous for the weak *frazzled* allele C1-62, a mild reduction in the number of commissural fibers is observed. Anterior as well as posterior commissures can be affected (arrows). (D) In mutant *commissureless*^{B204} embryos no commissures are formed. (E) Mutant *schizo*^{U112} embryos show a variable reduction in the number of commissural fibers. Note that the anterior commissure is affected preferentially (arrowhead). (F) In mutant *weniger*^{M454} embryos commissures are reduced. Here, the posterior commissure is perferentially missing (arrowhead).

kette, kästchen, kübel, spitz, Star, rhomboid, perle, cabrio, schmalspur, and *möchtegern*). *pointed* group embryos show a clear fusion of commissures and a frequent disruption of longitudinal connectives (Figs. 6 and 7). The midline glial cells are either reduced in number or fail to properly migrate inbetween anterior and posterior commissures (Fig. 7). In addition, all *pointed* group members have an relatively normal embryonic morphology. The PNS is characterized by a slight reduction in the number of sensory neurons (Fig. 6E) and defects in the trajectories of the motoneurons (data not shown).

Mutations in eight genes lead to an axon pattern phenotype initially described for *tramtrack* (Giesen *et al.*, 1997). In *tramtrack*-type mutations (*tramtrack, shroud, disem-*

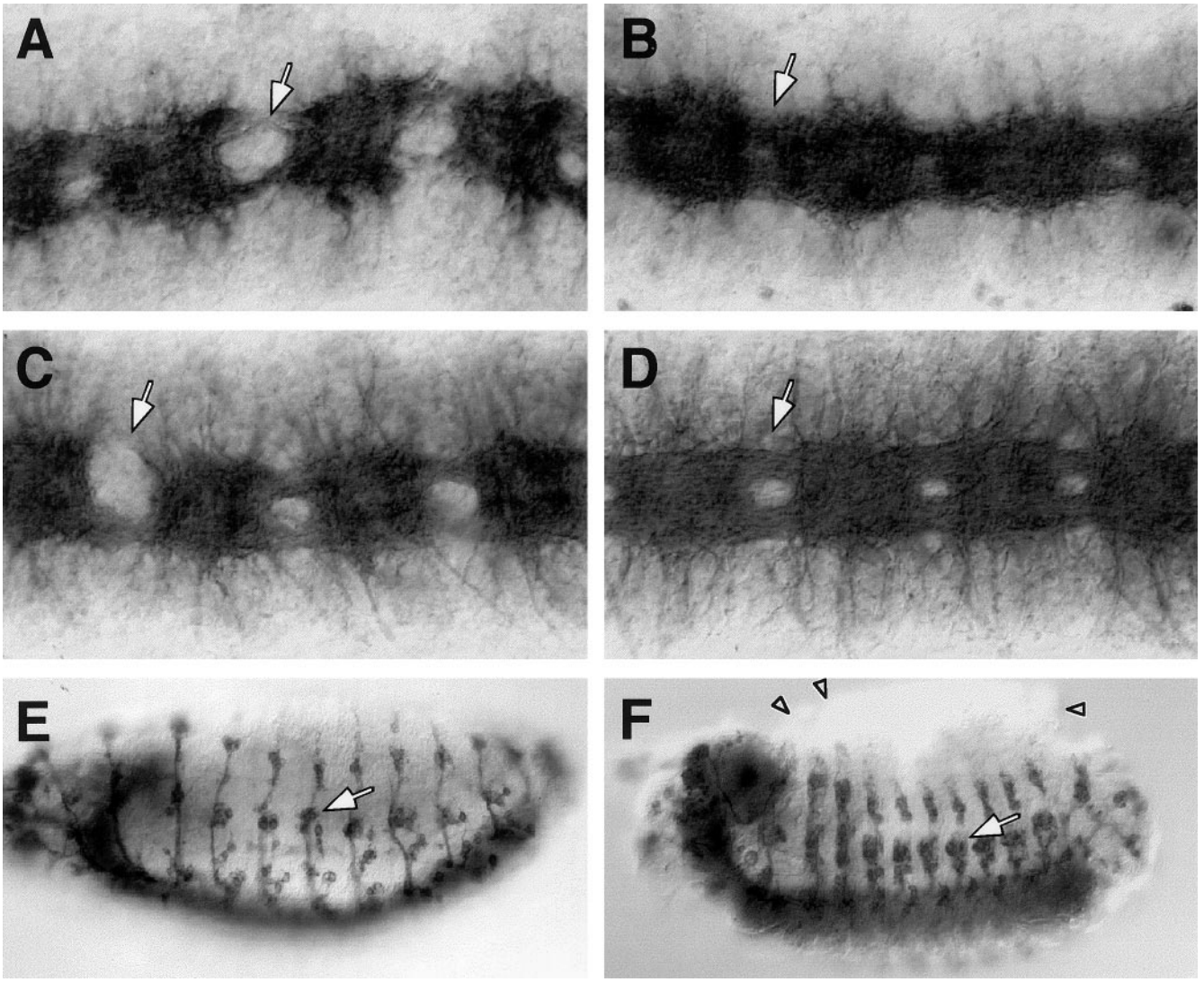


FIG. 6. Two groups of fused commissure mutations. (A–D) Frontal views of stage 15/16 CNS preparations. The CNS axon tracts have been labeled by AMb BP102 and subsequent HRP reaction. (E and F) Lateral views of stage 15/16 embryos stained for the presence of the 22C10 antigen, which is expressed in all sensory neurons. Anterior is to the left. (A) In *pointed* mutant embryos commissures appear fused and no space can be detected between anterior and posterior commissure. In addition, the connectives are thinner (arrow). (B) In a mutant *tramtrack* embryo commissures appear to be fused as well, but the connectives do not appear to be reduced (arrow). (C) Mutant *kette* embryos show a *pointed*-like phenotype. (D) Mutant *shroud* embryos show a similar fusion of commissures as mutant *tramtrack* embryos. (E) In *pointed* embryos the PNS is characterized by a slight reduction in the number of sensory neurons; the overall morphology of the embryo appears normal. (F) Mutant *shroud* embryos are characterized by a more compact overall body shape. Dorsal closure has not yet occurred (arrowheads).

bodied, *spook*, *shade*, *shadow*, *phantom*, and *rippchen*) commissures appear fused as well, but in contrast to *pointed* group mutations, connectives are not affected (Figs. 6B, 6D, and 7). A prominent feature of *tramtrack*-type mutations is the compact shape of the embryo. Indeed, most of the *tramtrack* group genes have been identified previously because of their common cuticle phenotype (Wieschas *et al.*, 1984; Jürgens *et al.*, 1984). In antibody whole-mount staining of stage 15/16 embryos it appears that the epidermis is displaced ventrally, leaving the dorsal side of the embryo open (Fig. 6F, arrowheads). Dorsal closure, however, does finally occur in these mutants (data not shown). The PNS often shows defasciculating axons and an increase in the number of sensory neurons (Fig. 6F). A detailed analysis of the *tramtrack* group genes will soon be presented (Giesen *et al.*, in preparation).

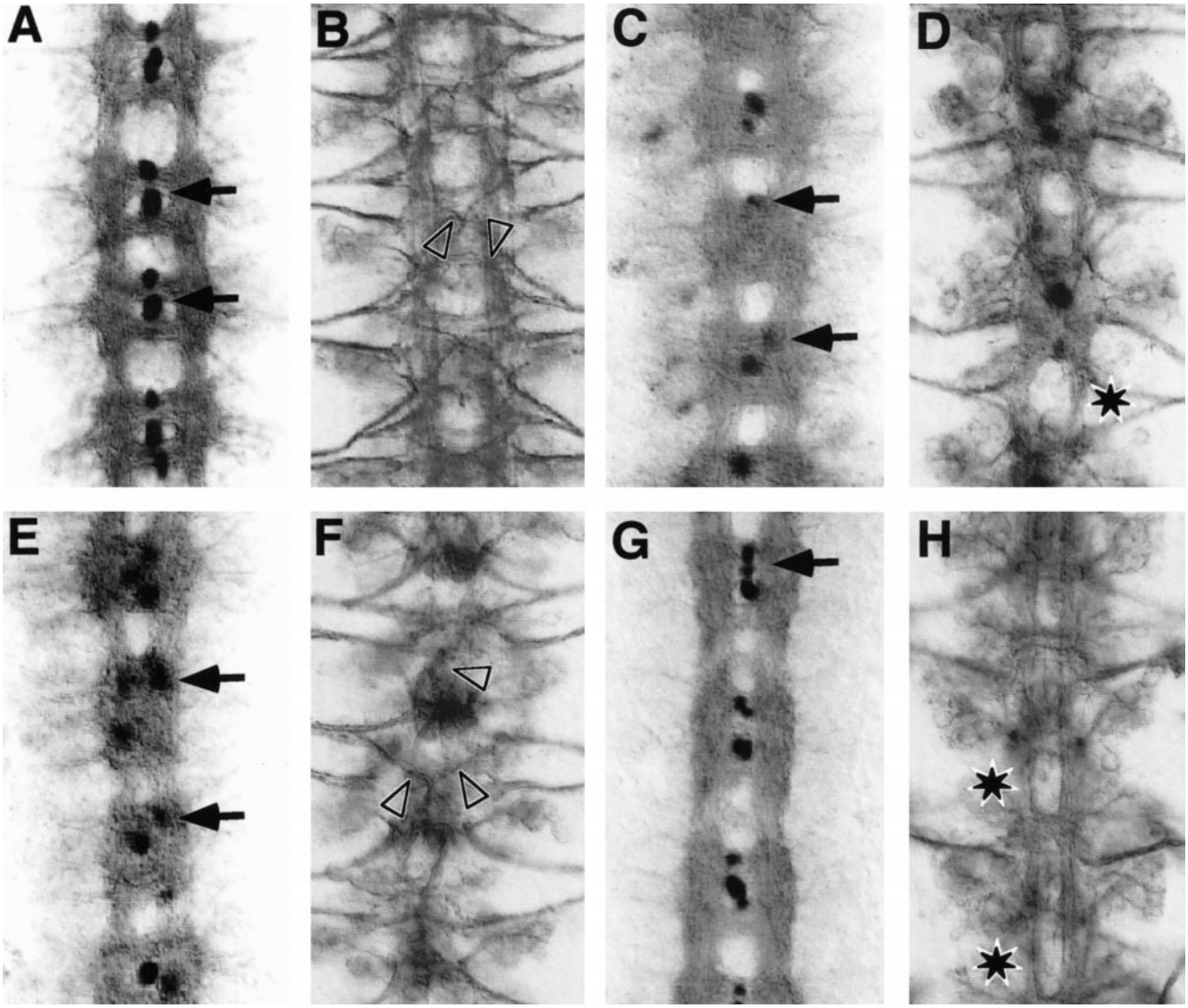
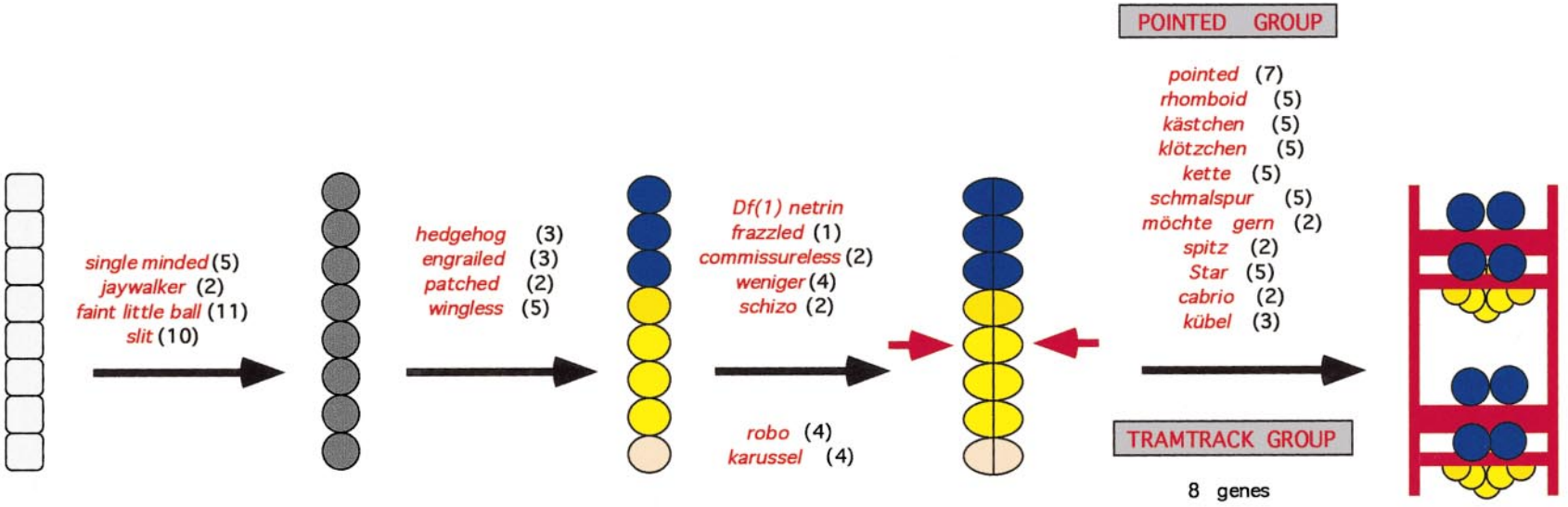
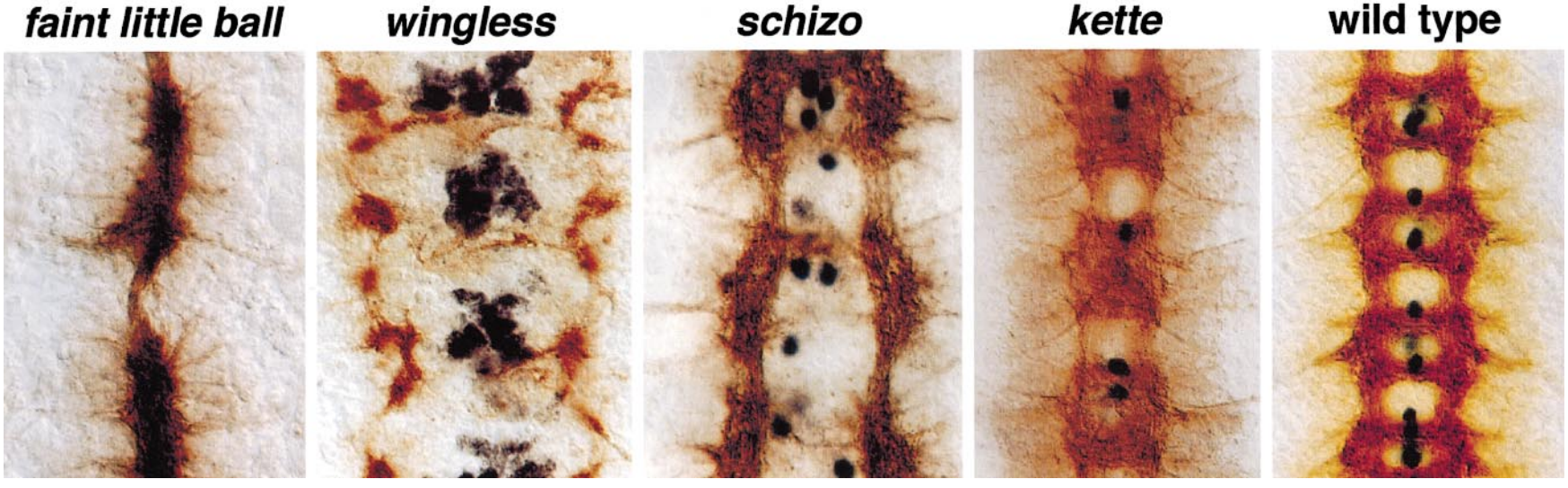


FIG. 7. Phenotypic analysis of *pointed* group mutations. Frontal views of dissected stage 16 CNS preparations. The CNS axon tracts have been labeled by MAb BP102 (A, C, E, and G) or MAb 22C10 (B, D, F, and H) and subsequent HRP reaction. β-Galactosidase expression in the midline glial cells is driven by the enhancer trap insertion *AA142* (A, C–E, and G). Anterior is up. (A and B) Wild type; (C and D) homozygous *kästchen*$^{B1\text{-}089}$ embryos; (E and F) homozygous *klötzchen*$^{E2\text{-}026}$ embryo; (G and H) homozygous *schmalspur*$^{D1\text{-}038}$ embryo. In wild-type embryos the midline glial cells migrate inbetween anterior and posterior commissures (arrows in A). In mutant *kästchen* embryos the number of midline glial cells is reduced compared to wild type and the cells are found dorsally to the commissures (arrows in C). In mutant *klötzchen* and *schmalspur* embryos, the midline glial cells shown abnormal migration behavior (arrows in E and G). MAb 22C10 labels the VUM axons (arrowheads in B and F). In mutant *klötzchen* embryos the VUM axons project irregularly; in addition longitudinal fascicles are missing. In mutant *kästchen* and *schmalspur* embryos 22C10 staining reveals fasciculation defects in the longitudinal connectives (asterisks in D and H).

FIG. 8. Gene controlling commissural development. The process of commissure formation can be divided into four steps (see text). The different genes required are indicated in the schematic drawing. Representative mutant phenotypes are shown below. The midline glial cells are labeled using the *AA142* marker. In *faint little ball* no more glial cells can be detected. In mutant *wingless* embryos, the number of glial cells is increased. In mutant *schizo* embryos a correlation between the number of glial cells and the number of axons crossing the midline can be observed. In mutant *kette* embryos the midline glial cells are reduced in number and do not differentiate properly, resulting in a failure to migrate inbetween anterior and posterior commissure. The fuzzy commissure mutations are thought to affect later developmental stages.


single minded (5)
jaywalker (2)
faint little ball (11)
slit (10)
hedgehog (3)
engrailed (3)
patched (2)
wingless (5)
Df(1) netrin
frazzled (1)
commissureless (2)
weniger (4)
schizo (2)
robo (4)
karussel (4)
POINTED GROUP
pointed (7)
rhomboid (5)
kästchen (5)
klötzchen (5)
kette (5)
schmalspur (5)
möchte gern (2)
spitz (2)
Star (5)
cabrio (2)
kübel (3)
TRAMTRACK GROUP
8 genes
Anlage of the CNS-midline
specification of midline cells
Formation of commissures
Separation of commissures


faint little ball
wingless
schizo
kette
wild type

DISCUSSION

In the present paper we report on a genetic screen aimed to identify all zygotically active genes required for proper *Drosophila* axon pattern formation. Here we focused on the development of commissural tracts in the ventral nerve cord. This is an interesting model system for studying axonal pattern formation, as it requires the action of long-range and short-range guidance mechanisms (Tessier-Lavigne and Goodman, 1996).

Following a saturating mutagenesis we have identified more than 700 mutations affecting the axon pattern in the embryo. The majority of the mutations disturb various aspects of neuronal differentiation and lineage, formation of the longitudinal axon tracts, axonal fasciculation, development of the PNS or condensation of the ventral nerve cord. About 20% of these mutations primarily affect the formation and morphogenesis of the commissures. Since commissure formation occurs in close relation to cells located at the CNS midline, we aimed to relate the different mutant commissural phenotypes to defects in these cells.

CNS Midline Functions during Development

The CNS midline cells in the vertebrate neural tube (floor plate cells) and in the *Drosophila* ventral nerve cord perform a number of similar functions. They are required for the establishment of the correct dorsal ventral polarity in the neural tube as well as having a function during the development of the mesodermal and ectodermal derivatives (Ericson *et al.*, 1996; Hynes *et al.*, 1995; Kim and Crews, 1993; Lüer *et al.*, 1997; Menne *et al.*, 1997; Munsterberg and Lassar, 1995; Porquie *et al.*, 1996; Roelink *et al.*, 1995; Yamada *et al.*, 1993). In particular, signals emanating from the midline organize the commissural axon pattern by either guiding growth cones toward the midline or preventing them from crossing the midline (Tessier-Lavigne and Goodman, 1996).

Model of Commissure Formation

Our analysis shows that defects in the commissural pattern can be related either to the absence of all or some midline cells or to defects in the presentation/perception of midline-derived organizing signals. Mutations in four zygotically active genes lead to a collapsed connective phenotype (Fig. 8). In these mutations the midline cells either are missing in late embryos (stage 15, *sim, flb*) or become dislocated ventrally (*slit,jay*). Two reasons might explain why we detected only a few genes required for this process. First, maternally derived factors might be required. These genes have necessarily escaped our screen. Second, genes required in many other developmental processes could lead to uninterpretable phenotypes. For example, some of the neurogenic genes, which we have discarded in our screen, are required for the activation of the *single-minded* gene (Martín-Bermudo *et al.*, 1995; Menne and Klämbt, 1994).

In *Drosophila* the CNS midline comprises glial and neuronal cell types (Bossing and Technau, 1994; Jacobs and Goodman, 1989; Klämbt *et al.*, 1991). The midline glial cell progenitors are located anteriorly and the neuronal progenitors are located in the posterior compartment of each segment. These cells delaminate from the ectoderm during germ-band extension where they form one mitotic domain (Foe, 1989). We have found that segment polarity mutants affect the specification of the different midline cell lineages (Fig. 8). It is thus possible that midline and ectodermal pattern formations occur at the same time. In mutant *hedgehog* embryos the posterior ectodermal compartment is missing and is replaced by the anterior portion. As a result mutant *hedgehog* embryos have a lawn of ventral cuticle (Martinez Arias, 1993). Likewise in the CNS the posterior lineages (= neuronal) are missing and appear to be replaced by anterior lineages (= glial). *hedgehog* normally represses the negative action of *patched* on the expression of *wingless*. In *patched* mutant embryos *wingless* expression is derepressed within a broad domain (for review see Martinez Arias, 1993). In the CNS midline of *patched* mutant embryos we observed the corresponding phenotype, loss of glial cells and increase in the number of midline neurons. The mutant *wingless* phenotype, however, suggests that additional signaling mechanisms are operating to specify the different midline lineages. In addition to the segment polarity genes other signaling mechanisms appear important. *Notch* is required to specify the different midline lineages. In *Notch* mutant embryos few midline glial cells are found, whereas in embryos expressing an activated Notch protein in the midline, additional midline glial cells are formed (Giebel, 1996).

In all mutations described above leading to a lack or reduction in the number of midline neurons we observed a reduction in the number of commissural tracts, whereas mutations which lead to a reduction in the number of midline glial cells showed a fused commissure phenotype. The correlation of axon pattern phenotypes and the different midline cell phenotypes suggests that the midline neurons are required to attract commissural growth cones, whereas the midline glial cells are required for the organization of commissural axons. However, since it is known that segment polarity mutations affect the specification of lateral neurons we cannot exclude a contribution of defects in contralateral projecting neurons to the axonal pattern phenotypes (Dormand and Brand, 1998; Patel *et al.*, 1989).

Formation of Commissures

Mutations in five genes lead to a reduction in the number of commissures without affecting cell fate at the midline (Fig. 8). *commissureless* was shown to be required to repress

the function of he roundabout repulsive receptor in lateral neurons (Kidd *et al.,* 1998a, b). Since *commissureless* is expressed in the midline and is not secreted this process appears to be contact dependent (Tear *et al.,* 1996). This is the only mutation we have isolated which leads to a complete loss of all commissural tracts. Mutations in *frazzled, schizo,* and *weniger* or the deletion of the *netrin* genes does not remove all commissural fibers (Harris *et al.,* 1996; Kolodziej *et al.,* 1996; Mitchell *et al.,* 1996). Further genetic analyses indicate that these genes are likely to act in several pathways (Hummel *et al.,* 1999).

Separation of Commissures

A large group of genes is needed for the separation of commissures and thus appears to be required for the development of the midline glial cells (Fig. 8). Contrary to midline specification and initial commissure formation this process occurs relatively late during embryogenesis and thus a maternal contribution is not likely to rescue a mutant phenotype. In addition, the separation of commissures requires not only the differentiation of the midline glial cells but also the differentiation of the midline neurons as well as the interaction of these two cell types for normal migration to occur (see Hummel *et al.,* 1999). This might explain the large number of genes identified.

The analysis of the mutations revealed two major phenotypic classes, the *pointed* and the *tramtrack* group. *pointed* and *tramtrack* mediate different aspects of glial development. In *pointed* mutants no glial differentiation occurs, whereas ectopic *pointed* expression results in ectopic glial differentiation (Klämbt, 1993; Klaes *et al.,* 1994). *tramtrack,* in contrast, does not interfere with actual glial cell differentiation but appears to be required for the repression of neuronal differentiation in these cells (Giesen *et al.,* 1997). The different members of the *pointed* and *tramtrack* groups could possible define new functions in these regulatory networks (Hummel *et al.,* 1999, Giesen *et al.,* in preparation).

Has Saturation Been Achieved?

We aimed to identify all zygotically acting genes required for commissure formation. In the screens of Nüsslein-Volhard, Wieschaus, and Jürgens about 17,000 lethal mutations were analyzed for cuticle defects (Jürgens *et al.,* 1984; Nüsslein-volhard *et al.,* 1984; Wieschaus *et al.,* 1984). With a similar or higher hit rate per chromosome we screened almost twofold the number of lethal hits (29,890), in order to compensate for the fact that due to the antibody staining, some mutations may have escaped our attention. Although we found 24 *Notch* alleles, we found only 2 *commissureless* or *schizo* mutations. For many genes we found only 1 EMS-induced allele each (*frazzled, tramtrack, perle*). For several fused commissure mutations we have not finished the complementation tests but some of these so far appear to be single hits as well. The average allele frequency for the genes described in this paper is 3.8 (Table 2). Although based on statistical reasons we have saturated the genome for mutations, the relative high number of single hits suggest that some genes may have escaped our screen.

An additional problem in studying nervous system development lies in the fact that partially redundant gene functions might be involved, as exemplified by the two *netrin* genes (Harris *et al.,* 1996; Mitchell *et al.,* 1996). Similarly, mutations in genes encoding cell adhesion proteins often do not result in a CNS phenotype, despite their strict regulation in time and space, suggesting the presence of redundant gene functions. The requirement of fasciclin I, or the related midline fasciclin, for the establishment of commissures is revealed only in embryos also mutant for the *abl* gene (Elkins *et al.,* 1990; Hu *et al.,* 1998). Since these proteins are expressed by CNS axons (Gertler *et al.,* 1989; McAllister *et al.,* 1992), they are likely to participate in the reception/transduction of an attractive signal. Further analysis of the reduced commissure mutations *schizo* and *weniger* revealed additional redundancy during commissure formation (Hummel *et al.,* 1999).

Despite the problem of redundancy we have identified a large number of genes with previously unknown functions during CNS development. In a first screen by Seeger *et al.* (1993), 250 mutations have been recovered from screening 13,529 mutagenized lines, which corresponds well with the 757 mutations we have isolated from screening 37,300 mutagenized lines. Here we have identified novel gene functions that escaped in previous screens and related them to commissure formation.

Conclusions

Our genetic analyses have uncovered a distinct set of genes, many of which were previously unknown, required for the development of the commissural axon patter in *Drosophila.* Following phenotypic and genetic analyses (see Hummel *et al.,* 1999) we integrated these gene functions into a working model of commissure formation. The ongoing *Drosophila* genome project will accelerate the molecular analysis of a large number of the genes identified in this work, which soon will lead to a better understanding of the molecular networks underlying these aspects of CNS development.

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